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2) Zakataeva NP, Aleshin VV, Tokmakova IL, Troshin PV, Livshits VA.
The novel transmembrane Escherichia coli proteins involved in the amino acid efflux.
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Thank you,
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The novel transmembrane *Escherichia coli* proteins involved in the amino acid efflux

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Abstract A novel gene of *Escherichia coli, rhtB*, has been characterized. Amplification of this gene provides resistance to homoserine and homoserine lactone. Another *E. coli* gene, *rhtC*, provides resistance to threonine. The homologues of RhtB are widely distributed among various eubacteria and archaea, from one to 12 copies of family members that differ in their primary structure were found in the genomes. Most of them are genes that encode hypothetical transmembrane proteins. Experimental data that indicate participation of the *rhtB* product in the excretion of homoserine have been obtained.

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Key words: Efflux; Transport; Transmembrane protein; Homoserine; Threonine

1. Introduction

Sequencing of genomes of eubacteria, archaea and eukaryotes revealed a large amount of gene sequences that encode transmembrane proteins. The majority of them are transport proteins that belong to the two largest protein superfamilies, the ATP-binding cassette (ABC transporters) and the major facilitator superfamily (MFS). The latter superfamily includes proteins that carry out the transport of substrates by means of a transmembrane electrochemical proton gradient. The compounds that are transported by proteins of these two superfamilies include simple sugars, oligosaccharides, inositols, drugs, amino acids, nucleosides, organophosphate esters, Krebs cycle metabolites and a large variety of organic and inorganic anions and cations [1,2]. Many of the proteins that transport substrates inside of the cells are well-studied. The efflux proteins are less investigated, although these proteins have been shown to determine resistance to toxic compounds and to participate in maintenance of the optimal intracellular concentration of metabolites and in the excretion of some regulatory molecules [3–5].

Homoserine, a metabolic precursor of threonine and methionine, is classified with important regulators of various bacteria. In cells of *Escherichia coli*, homoserine inhibits $NADP^+$-specific glutamate dehydrogenase (E.C. 1.4.1.4), the enzyme that catalyzes the primary reaction of the ammonium assimilation [6]. Homoserine lactone, a derivative of homoserine, was shown to activate expression of the σ^S subunit of RNA polymerase [7]. The σ^S subunit determines the transcription-specificity of the gene ensemble that acts during starvation or at the entry of the *E. coli* cells into the stationary growth phase [8].

In this paper, we present the identification of the *E. coli* genes *rhtB* and *rhtC*. The product of the *rhtB* gene carries out the efflux of homoserine and homoserine lactone, the product of the *rhtC* gene conducts the efflux of threonine. The significance of bacterial amino acid efflux proteins is discussed.

2. Materials and methods

2.1. Bacterial strains and culturing conditions

In this study, we used the following *E. coli* strains: N99 (su$^-$ *lac galKT* IN (*rrnD-rrnE*) *rpsL*) (the collection of GNIIGenetika); MG442 (F$^-$ *thrA442 ilvA442*) [9], C600 (*thi1 thrB1leu6 lacY1 supE44 tonA21 rpsL rfbD1*) [10] and NZ10 (C600 *leu*$^+$). We used the multicopy vectors pBluescript KS$^+$ (Stratagene), pUC21 [11], pUK21 [11], pACYC177 [12], pACYC184 [12] and the plasmid pAL4. The latter is the derivative of pBR322 [13] that included the cloned gene *thrA* for the bifunctional protein aspartate kinase (EC 2.7.2.4) I-homoserine dehydrogenase (EC 1.1.1.3) I, the first enzyme in the homoserine and threonine biosynthesis pathway.

Growth media M9 and LB [14], amino acids and their analogues and antibiotics were provided by Sigma.

2.2. Genetic constructing and characterizing phenotypes

The cloning of the *rhtB* gene was conducted using the phagemid Mu d5005, according to the technique described in [15]. As a DNA donor of bacterial chromosomes, we used the strain MG442 that is lysogenic for Mu *cts*. The phagemids that had insertions were selected using the minimal nutritional medium M9 that contained kanamycin and homoserine (10 mg/ml) or threonine (50 mg/ml).

The integration of the obtained insertion mutation *rhtB*::*cat* into the chromosome of strain N99 was carried out by means of the method described by Parker and Marinus [16]. The mutation *rhtB*::*cat* was introduced to different strains by transduction using the phage P1 *vir*.

All DNA manipulations were performed according to standard procedures [17].

The resistance of different strains to amino acids and their analogues was determined as follows. The cells (10^3–10^4) that were grown in the liquid minimal nutritional medium were placed onto agar minimal nutritional media that contained essential growth factors and differed in the concentration of the corresponding amino acid or its analogue.

The intracellular amino acid pools were determined according to [18]. The results were calculated in relation to the dry weight (DW) of cells.

2.3. Sequencing of DNA and further analysis

Sequencing of DNA was conducted according to Sanger's method [19]. The sequences were checked for the presence of transmembrane segments by means of the PHDhtm program [20].

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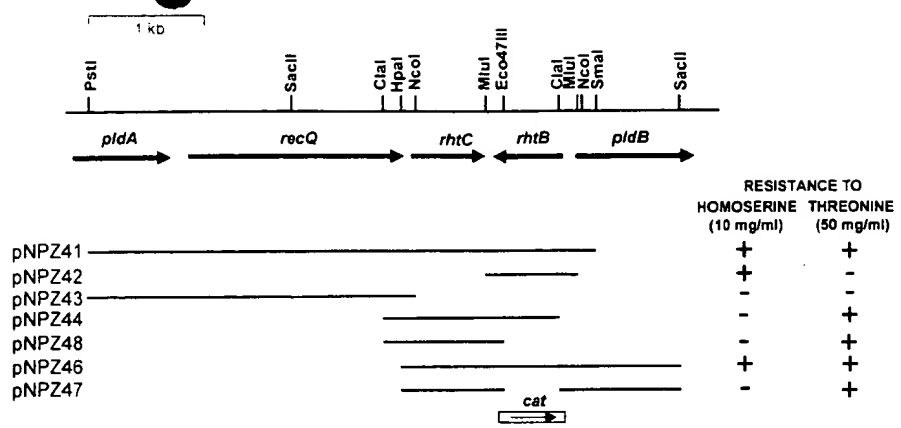

Fig. 1. Physical map of the 86 min region of the *E. coli* chromosome. The subcloned regions and the designations of the corresponding plasmids and phenotypes are presented below.

3. Results

3.1. Cloning and identification of the rhtB gene

Exogenetic homoserine lactone, homoserine and threonine suppress the growth of *E. coli* in minimal nutritional media. This is possibly occurring due to blockage of the ammonium assimilation or/and due to activation of the σ^S expression. Amplification of genes for components of systems that eliminate antibiotics, organic solvents and metal ions from the cell increases the resistance of bacteria to these substances [21–23]. In view of this, we found that cloning (using a multicopy vector) of an *E. coli* chromosomal DNA fragment from the 86 min region resulted in resistance of cells to homoserine lactone, homoserine and threonine.

Initially, we performed in vivo cloning of the DNA fragment from the 86 min region, using the phagemid Mu d5005. The cloned fragment included the *recQ* gene and two open reading frames (ORFs), o128 and f138 (GenBank accession number M87049) [24]. Subcloning of different regions of this fragment in multi-copy vectors and analysis of the obtained constructions showed that the minimal size of the fragment that provides resistance to high concentrations of homoserine (more than 10 mg/ml) is 0.8 kb (plasmid pNPZ42, see Fig. 1). This minimal fragment included ORF f138 and the upstream sequence of 348 bp. Note that plasmid pNPZ44 that contained ORF f138 with only 160 5′-flanking nucleotides did not provide resistance to high concentrations of homoserine. We found that the upstream sequence in frame with f138 did not contain a stop codon. Moreover, one of the ATG codons of this sequence is preceded by a ribosome-binding site (62171–62166 in M87049). We designated the extended ORF with an additional 67 amino acid residues (62160–61546 bp) the *rhtB* gene (resistance to homoserine and threonine). Conversely, the fragment that included ORF o128 for the hypothetical protein YigJ (plasmid pNPZ48, Fig. 1) was shown to provide no resistance to high concentrations of homoserine, but conferred resistance to high concentrations of threonine (50 mg/ml).

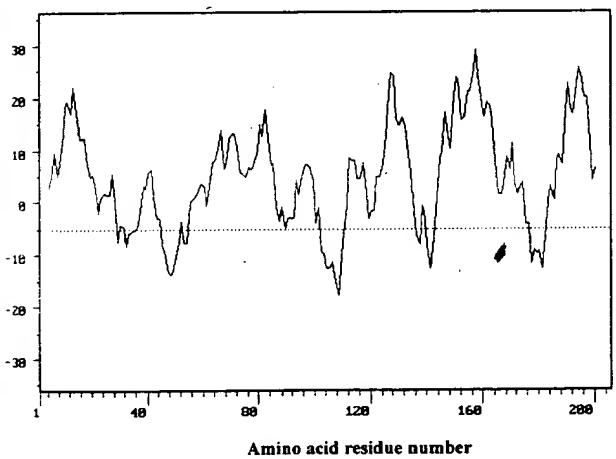

Fig. 2. Hydropathy plot of RhtB. The algorithm of Kyte and Doolittle [33] was used with a window span of nine.

3.2. The RhtB protein belongs to a new family of transmembrane proteins

Based on the predicted amino acid sequence, analysis revealed that protein RhtB is highly hydrophobic and contains

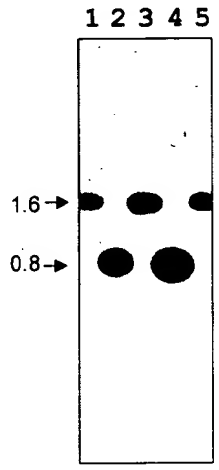

Fig. 3. Southern blot of genomic DNA that indicates the state of the locus *rhtB* in different strains. Strains 2 and 4 contain the wild-type allele of the gene *rhtB* (the genomic DNA of N99 and the plasmid pNPZ46, respectively); strains 1, 3 and 5 include *rhtB*::*cat* (the genomic DNA of N99 *rhtA*::*cat*, the plasmid pNPZ47 and the genomic DNA of NZ10 *rhtA*::*cat*, respectively). Genomic and plasmid DNA were digested with *Mlu*I and hybridized using the 0.8 kb *Mlu*I-*Mlu*I *rhtB*-containing fragment (see Fig. 1) as a ^{32}P-labelled probe.

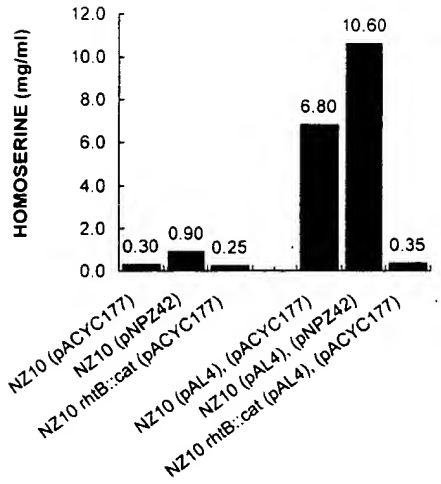

Fig. 4. The effect of the gene *rhtB* on accumulation of homoserine in the medium. Cells were cultivated in minimal nutritional medium for 48 h. Results are the mean for at least three independent determinations.

six predicted transmembrane segments (Fig. 2). A PSI-BLAST [25] search for homology in the non-redundant database at the NCBI and a gapped BLAST [25] search in unfinished microbial genomes revealed more than 60 proteins that are homologous to RhtB in various eubacteria and archaea [26]. All these proteins include approximately equal amounts of amino acid residues and they are hydrophobic and share a similar profile for transmembrane segments. They are not included in any described clusters of orthologous groups (COGs) [27], but some of them are recently included in the UPF0048 family. We established three conservative motifs that characterize these proteins as a unified protein family [26].

Exceptional representatives that are depleted of the first motif Pro-Gly-Pro were found among RhtB-related proteins obtained from databases. These are the hypothetical proteins MlgA of *Shewanella colwelliana* (GenBank accession number X67020) and YigJ of *E. coli* (GenBank accession numbers M87049 and AE000458). However, there is not any stop codon upstream of the initiator codon of MlgA. By adding 24 triplets upstream of the new putative initiator codon, a pair Pro-Gly was found in the position of the first motif. Moreover, second and third motifs in the extended protein are here situated at a canonical distance from the N-terminus.

Protein YigJ that is encoded by ORF o128 of *E. coli* is homologous to RhtB. When cloned by the multi-copy vector (Plasmid pNPZ48, Fig. 1), the gene for YigJ provides resistance to high concentrations of threonine. However, we did not observe the first conservative motif in the transcription of o128. The Pro-Gly-Pro sequence was found, if translation was assumed in another frame. Sequencing of this region showed a frameshift at position 61214, as related to M87049. In the revised sequence, the Pro-Gly-Pro motif is situated in frame with the third conservative motif at canonical distances both from the third motif and the new predicted N-terminus. These data agree with the revision of YIGJ_ECOLI in the recent SWISSPROT release (Rood, K.E., unpublished data). A novel protein that was designated by us as RhtC includes 206 amino acid residues, contains three afore-mentioned conservative motifs that are characteristic of the family and is likely to participate in the excretion of threonine.

3.3. *The RhtB protein is responsible for transport of amino acids and their analogues out of the cell*

Most of the RhtB family members are transmembrane proteins, whose function has not yet been determined. A representative of this family, the protein LysE of *Corynebacterium glutamicum*, is now the only identified transporter that conducts efflux of an amino acid (lysine) [28]. The amplification of another homologue, the afore-mentioned protein RhtC of *E. coli* (in earlier sources, YigJ), provides resistance to threonine. The function of the *rhtB* gene was studied in isogenetic *E. coli* strains that included different alleles of this gene. To accomplish this, we conducted insertion inactivation of this gene. We substituted the major part of the encoding region of the gene *rhtB* (Fig. 1) by the gene *cat* (Cm^R) of the plasmid pACYC184. The integration of the insertion mutation *rhtB*::*cat* into the chromosome is described in Section 2. Substitution of the wild-type allele of the gene *rhtB* by insertion mutation was confirmed by genetic mapping and Southern hybridization (Fig. 3).

Inactivation of the *rhtB* gene on the chromosome was found to have no effect on the viability of bacteria. However, the wild-type allele of the *rhtB* gene and its inactivated variant *rhtB*::*cat* had different effects on accumulation of homoserine in the medium. Incorporation of the plasmid that contained the wild-type allele of the gene *rhtB* into the producer strains of homoserine NZ10 and NZ10 (pAL4) resulted in a considerable increase in the accumulation of this amino acid in the medium. Conversely, in the strains with the inactivated gene *rhtB*::*cat*, a decrease in the accumulation of homoserine in the medium was observed (Fig. 4).

Determination of the intracellular homoserine pools of

Table 1
Effects of the amplification and inactivation of the *rhtB* gene on the resistance of *E. coli* cells to some amino acids and amino acid analogues

Substrate	Minimal inhibitory concentration (μg/ml)		
	N99 (pACYC177)	N99 (pNPZ42)	N99 *rhtB*::*cat*
(pACYC177)			
L-Homoserine	500	40000	250
L-Homoserine lactone hydrochloride	500	>5000	250
DL-β-Hydroxynorvaline	50	2000	25
L-Serine	5000	15000	5000
4-Aza-DL-leucine	50	100	50
L-Glutamic acid γ-hydrazide	5	10	5
L-Threonine	30000	50000	30000
L-Histidine	5000	5000	5000
L-Valine	0.5	0.5	0.5

strains that included different alleles of the *rhtB* gene showed that amplification of the *rhtB* gene appreciably decreased the intracellular pool of this amino acid, whereas inactivation of this gene, conversely, increased the pool to some extent. The intracellular pools of homoserine (calculated as mean data from three independent experiments) were, respectively, 47.6, 2.0 and 58.2 nmol/mg (DW) in the strains NZ10 (pACYC177), NZ10 (pNPZ42) and NZ10 *rhtB*::*cat* (pACYC177). Comparable intracellular pools in NZ10 (pACYC177) and NZ10 *rhtB*::*cat* (pACYC177) indicate that the RhtB protein influences the accumulation of homoserine in the medium rather than synthesis of this amino acid in the cell.

The wild-type strains, the null-mutant for the *rhtB* gene and the strain with the amplified wild-type allele of the gene *rhtB* considerably differed in the resistance to various amino acids and their analogues (Table 1). These data are evidence that protein RhtB carries out the excretion of not only homoserine, but also homoserine lactone and some other amino acids and their analogues and increases resistance to them. The inactivation of the *rhtB* gene on the bacterial chromosome, conversely, results in a decrease in the level of bacterial resistance to L-homoserine, homoserine lactone and DL-β-hydroxynorvaline. These effects of the *rhtB* gene on the phenotype are indicative of participation of the product of the *rhtB* gene in the excretion of homoserine and, probably, some other amino acids.

4. Discussion

The RhtB family consists of two subfamilies and includes more than 60 representatives of eubacteria and archaea. This number increases along with sequencing of more genomes. Most of the members of this family are hypothetical transmembrane proteins. The proteins LysE, RhtB and RhtC are involved in the excretion of lysine, homoserine (homoserine lactone), threonine and some amino acid analogues. The four remaining paralogues of this family that are found in the genome of *E. coli* [26], according to our unpublished data, are also able to excretion of other substrates rather than homoserine and threonine.

The proteins that carry out excretion of compounds out of the cell comprise a considerable part of the genomes of eubacteria and archaea. The efflux of toxic substances is reasonable, whereas the significance of the excretion of amino acids is still unclear. There are grounds to assume that these systems are necessary for the communicative function and quorum-sensing effects in microbial populations. High concentrations of the substances to be excreted in the medium indicate attainment of a certain population density, which is the signal for initializing global cellular response systems, such as, e.g., cellular differentiation and social behavior.

The role of amino acids and their derivatives as transmitters in intercellular communication was shown in many eukaryotic and some prokaryotic systems. A specific subset of amino acids with the prevalence of tyrosine, proline, phenylalanine and isoleucine that is excreted by all cells of *Myxococcus xanthus* is a signal for the formation of fruit bodies in this myxobacterium, started from attainment of the threshold concentration of these amino acids in the medium [29]. The acylated homoserine lactones that are synthesized and then excreted into the medium enable *Pseudomonas aerogenosa*, *Vibrio fischeri* and other microorganisms to control their population density [5]. Thus, for example, the cells of *V. fischeri* begin to produce luminescence, when their population density reaches a certain level. The content of homoserine lactone (apoinductor) in the medium reaches the threshold concentration and the expression of the *lux* genes that are responsible for luminescence is stimulated [5]. In *E. coli*, homoserine lactone is a substance that induces the entry of cells into the stationary growth phase [7].

Furthermore, the genes for some members of the RhtB family, whose function has not yet been experimentally determined, are situated near the operons for biosynthesis of homoserine lactones and non-canonical amino acids or are included in them. For example, the gene *yggA* of *Aeromonas salmonicida* is likely to be included in the operon for the *lux* genes [30]. The protein CmaU of *Pseudomonas syringae* is encoded by a gene of unknown function that is included in the biosynthetic gene cluster for coronamic acid, a precursor of coronatine (2-ethyl-1-aminocyclopropane 1-carboxylic acid) [31]. Coronatine is excreted into the medium and acts similarly to the growth hormones of plants. Interestingly, among the members of the afore-mentioned family of transmembrane proteins described by us, a sodium-dependent serotonin transporter of *Bacillus* sp. was found (PID:d1032489). Serotonin (5-oxytryptamine, an analogue of tryptophan) is known to be an important evolutionarily conservative animal hormone and neuromediator that influences the growth rate, the cell aggregation and formation of the extracellular matrix in some microorganisms [32]. Apparently, the proteins of the RhtB family comprise the evolutionarily conservative transporters that have long been used for the excretion of various amino acids and their derivatives out of cells.

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